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Stabilisation/Solidification Treatment and Remediation

Advances in S/S for Waste and Contaminated Land

Edited by

Abir Al-Tabbaa
Department of Engineering, University of Cambridge, United Kingdom

Julia A. Stegemann
Department of Civil and Environmental Engineering, University College London, United Kingdom

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Stabilisation/Solidification Treatment and Remediation – Al-Tabbaa & Stegemann (eds)

Stabilization of chromium by reductase enzyme treatment

K.S.M. Rahman & M.A.V. Murthy
Brookhaven National Laboratory, New York, USA

ABSTRACT: Hexavalent chromium (Cr(VI)) is highly toxic, and a major heavy metal contaminant in the environment. An important strategy for bioremediating Cr(VI) is to microbiologically reduce it to less toxic Cr(III). One of the major routes of bacterial chromate reduction is enzymatic reduction mediated by chromate reductase after its uptake inside the cell. While the enzymatic pathway is common in aerobic bacteria, it probably also occurs anaerobically. To gain more insight about this pathway, we investigated the reduction of Cr(VI) by a highly resistant *Bacillus* species. Reduction depended on which of three organic substrates was used, i.e., glucose, lactate and acetate at 0.1 to 2.0 mM, and the rate of reduction decreased with increasing concentration of chromate. Because sulfhydryl sites are known to be active sites for enzyme reductase, bacterial growth and reduction of Cr(VI) by *Bacillus* in the presence of varying concentrations of sulfate and thiosulfate were investigated. While changes in sulfate did not affect the reduction rate, raising the thiosulfate concentration in the medium from 0.05 to 1.0 mM markedly increased the reduction rate. Thiosulfate enhanced the reduction of Cr(VI), probably by accelerating the biosynthesis of chromate reductase enzymes, although other mechanisms may be involved. Our results show that the enzymes or other substances mediating the reduction reside mostly in the cytoplasm. This reductase enzyme could be extracted and applied sites contaminated with Cr(VI), to convert it to Cr(III), which would prevent leaching of the pollutant to groundwater.

1 INTRODUCTION

Chromium is one of the major heavy-metal contaminants in the environment. Many industries release chromium during their operations, including the metal-finishing industry, petroleum refining, leather tanning, iron- and steel-production, inorganic-chemical production, textile manufacturing, and pulp-producing processes. In general, the toxicity of heavy-metal contaminants may be related to their chemical speciation. For chromium, the oxidized hexavalent species present in complex anions, such as chromate (CrO_4^{2-}), bichromate ($HCrO_4^-$), and dichromate ($Cr_2O_7^{2-}$), is highly toxic, and is likely to be a carcinogen and a mutagen (Connent & Wetterhahn, 1983). However, reduced Cr(III), which readily forms insoluble oxides and hydroxides, [$Cr(OH)_3$], is regarded as less toxic or non-toxic to organisms (Tandon et al. 1978, Rai et al. 1987). Consequently, the environmental toxicity of chromium arises mainly from the presence of Cr(VI). Because the Cr(III) species is not poisonous to humans, the reduction of Cr(VI) to Cr(III) may constitute a potentially valuable mechanism for remediating Cr(VI) toxicity.

Both abiotic and biotic processes can reduce environmental Cr(VI) to Cr(III) (Smillie et al. 1981, Wang & Xiao 1995, Lovley & Phillips 1994). The redox potential (Eh) of the immediate environment, its pH, and the presence of reductive molecules are some important factors controlling the rate of abiotic reduction. Reactive sulfur species, such as hydrogen sulfide and thiols, probably play a crucial role in reducing Cr(VI) under reducing conditions (Smillie et al. 1981).

Bacterial reduction is a significant pathway for reducing Cr(VI) to Cr(III), both anaerobically and aerobically. Since the first report in the 1970s of the isolation of chromium-reducing *Pseudomonas* strains from chromate-contaminated sewage sludge (Romanenko & Koren'kov 1977), several more chromate-reducing bacteria have been identified. They include additional strains of *Pseudomonas*, as well as strains of *Micrococcus*, *Escherichia*, *Enterobacter*, *Bacillus*, *Aeromonas*, and *Achromobacter* species (Kavasnikov et al. 1985, Gvozdyak et al. 1986, Horitsu et al. 1987, Bopp & Ehrlich 1988, Wang et al. 1989, Ishibashi et al. 1990). The mechanisms by which these microorganisms reduce Cr(VI) vary and are species-dependent. Essentially, there are three major routes of reduction. The first two are anaerobic only; the third occurs mostly under aerobic conditions, but also can occur under anaerobic conditions.

In one anaerobic pathway, bacteria use Cr(VI) as a terminal electron acceptor in their respiratory chains, with organic substrates as the reductants; the list encompasses many facultative anerobes, including

P. aeruginosa (Gvozdyak et al. 1986), *B. subtilis* (Gvozdyak et al. 1986), *P. fluorescens* (Bopp & Ehrlich 1988), *E. cloacae* (Wang et al. 1989), and some sulfate-reducing bacteria (Fude et al. 1994, Tebo & Obraztsova 1998). A second feasible pathway under reducing conditions involves the initial production of reductants, such as hydrogen sulfide, followed by their reaction with Cr(VI) (Smillie et al. 1981, Lupton et al. 1991).

In a third pathway, bacteria use the soluble metal reductases present in their cytoplasm to reduce Cr(VI) enzymatically (Horitsu et al. 1987, Ishibashi et al. 1990, Shen & Wang 1993). Because this reaction occurs within the cytoplasmic milieu, it can take place under anaerobic and aerobic conditions. Once Cr(III) is generated by microbial processes, it then can be expelled to the outer surface of the cell and bind to the electronegatively charged surface-functional groups present there (Beveridge & Murray 1976), forming stable nucleation sites for further precipitation of Cr(III) mineral phases (McLean et al. 1999).

Because of the complexity of the enzymatic pathway, details of the mechanism are far from clear. To gain more insights about this pathway, we investigated growth and Cr(VI) reduction by an aerobic *Bacillus sp.* obtained from American Type Culture Collection (ATCC strain 700729). We used this *Bacillus* strain because of its resistance to high concentrations of chromium. It was first isolated from chromium contaminated tannery wastewater. Generally, *Bacillus* species withstand extreme conditions because of their ability to form spores, among other characteristics. Studies were conducted with different organic substrates (glucose, lactate, and acetate), representing different types of common metabolic end-products with simple structures, and with two types of sulfur sources. Sulfhydryl groups of enzymes and proteins are known to play a crucial role in cellular redox processes (Miller et al. 1989, Rabuck et al. 1990, Ellis & Poole 1997).

Stabilization and solidification technologies involving cement materials (S/S) have been used for many decades, and remain a viable option for the treatment and disposal of chemically hazardous materials. Major attractions of S/S that lead to its being described as the best demonstrated available technology (BDAT) include low material and operating costs, ease of use, and capacity to contain waste and prevent its migration into the natural environment. Addition of cement materials to organic or inorganic waste can reduce the mobility of many contaminants by chemical and physical mechanisms. A disadvantage of S/S is that it prevents natural attenuation and microbial biodegradation.

The most commonly used binder for waste S/S is ordinary Portland cement (OPC) (Idachaba et al. 2003). S/S has previously been put forward as a possible treatment for chromium wastes. Idachaba et al. (2003) studied the best working combination of chromium nitrate and cement. Kindness et al. (1994) noticed that in the presence of blast furnace slag, Cr(VI) was reduced to Cr(III), which can precipitate a stable and insoluble form of $Cr(OH)_3$. According to Allan & KuKacka (1995), blast furnace slag seems to be able to exert a solubility control on chromium. Otomoso et al. (1995) studied the S/S mechanism involving chromium and tricalcium silicate. Dermatas & Meng (2003) effectively immobilised Cr(III) by quicklime-fly ash treatment. So far no biological method for Cr S/S has been reported.

Enzyme technology is an established technique, and enzymes produced by microorganisms could be used for this purpose without introducing microorganisms into the environment. The latter is a particular concern for genetically modified microorganisms.

2 MATERIALS AND METHODS

2.1 *Media*

A mineral salt medium (MSM) was formulated (modified from Guha et al. 2001) and optimized for use under the conditions of the Cr(VI) reduction experiment. The MSM had the following composition: 6 g Na_2HPO_4, 3 g KH_2PO_4, 0.5 g Na Cl 1 g NH_4Cl, 1 mL of 1 M $MgCl_2$, 1 mL of 1 M Na_2SO_4, 0.5 g Tryptone, 1 L distilled water, adjusted to pH 7.

2.2 *Microorganism*

Bacillus sp. ATCC 700729 was used in the experiment. The cells to be used as inocula were routinely sub-cultured in shake flasks containing MSM with glucose as the energy source.

2.3 *Reduction experiments with different substrates*

The aerobic reduction experiments were carried out in 1 L Erlenmeyer flasks containing 500 mL MSM. Cell suspensions (ca. 7×10^3 cells/mL) were added to these flasks along with 1% glucose, 1% sodium citrate or 1% sodium lactate, and various concentrations of chromate (0.1, 0.2, 0.5, 0.75, 1.0 and 2.0 mM). Cultures were incubated at 37°C without shaking. Samples were taken for Cr(VI) analysis and for estimating bacterial growth at various times afterwards. Culture aliquots were plated on nutrient agar (Sigma) without Cr(VI) to assess cell viability. For each treatment, cell-free control flasks were prepared to monitor whether abiotic chromate reduction occurred. All experiments, including the abiotic controls were conducted in duplicate.

Reduction of Cr(VI) in the presence of different concentrations of sulfate and thiosulfate was investigated by adding 0.05 or 1.0 mM of the sulfur compounds to the culture. Because thiosulfate forms a precipitate with

Cr(VI) during autoclaving, we first filter-sterilized the thiosulfate and then added it to the medium and the Cr(VI) that had been routinely autoclaved.

2.4 *Bacterial growth*

Bacteria were counted by the Pour plate technique. The samples were serially diluted and plated on nutrient agar and incubated at 37°C for 24 h. The number of bacteria growing on the agar surface was counted and expressed as colony Forming Units (CFU) per mL.

2.5 *Cr(VI) estimation*

Chromate [Cr(VI)] in the liquid fraction of each treatment was analyzed at various times by aseptically removing an aliquot of the liquid followed by centrifugation at 13,000 X g for 5 minutes in 2.0 mL microcentrifuge tubes. Cr(VI) in the supernatant was then measured colorimetrically at 540 nm using the diphenyl carbazide method (APHA 1989).

2.6 *Cell fractionation*

Cells were grown in MSM at 37°C and harvested at mid-exponential phase by centrifugation at 4000 X g. They were washed three times by centrifugation in HEPES buffer (pH 7), resuspended in 10 mL of the same buffer, and kept in an ice bath. Cells were mechanically ruptured using an ultrasonicator (Cole-Parmer, model 8845-5). The suspension then was centrifuged at 12,000 X g to pelletize the unbroken cells. The supernatant was spun at 150,000 X g for 2 h at 4°C. This high speed supernatant (S_{150}) was retained as the cytoplasmic fraction, while the pellet obtained from this step was resuspended in 10 mL of buffer and used as the membrane fraction. The Cr(VI) concentration in the cell membrane and cytoplasmic fractions were adjusted to 0.1 and 0.2 mM, and Cr(VI) reduction was estimated at 0 h and 12 h after adding Cr(VI).

3 RESULTS AND DISCUSSION

3.1 *Growth and chromium reduction with different organic substrates*

Figure 1 gives the time-series measurements for the growth of the bacterium with different organic substrates in the presence of different concentrations of Cr(VI).

In glucose medium with no Cr(VI) present, the bacteria grew rapidly from an initial population of 7×10^3 CFU/mL to 6.6×10^5 CFU/mL in 24 h. The stationary phase was reached after about 96 h. Increasing the concentration of Cr(VI) in the medium significantly reduced bacterial growth (Figures 1A & 1B). At concentrations of 0.1 and 0.2 mM, Cr(VI) was completely reduced after 96 and 168 h respectively.

The kinetics of growth and Cr(VI) reduction in the lactate medium were similar to those in the glucose medium with an increase of the population from 7×10^3 CFU/mL to 2.8×10^5 in the first 24 h period with no added Cr(VI). The rates of Cr(VI) reduction varied with the initial level of added Cr(VI). At 0.1 mM initial Cr(VI), complete reduction was achieved within 72 h, whereas it took 168 h to completely reduce 0.2 mM Cr(VI). With acetate as the organic electron acceptor, the bacterial count also increased to a maximum of 3.9×10^5 during the first 24 h incubation period with no Cr(VI) present.

However, when Cr(VI) was added, Cr reduction was noticeably lower than when the microbe was cultured on glucose and lactate. We calculated approximate relative rates for Cr(VI) reduction at different intervals using a polynomial fit for the curves for time vs percent reduction of Cr(VI) (Table 1).

As shown in Figure 1 and this table, the maximum efficiency of reduction was observed in glucose followed by lactate and then acetate. Schmieman et al. (1997) observed the growth of a mixed bacterial culture in the presence of Cr(VI) with acetate as the substrate, but recorded no reduction of Cr(VI). By contrast, the species of *Bacillus* we used tolerated Cr(VI) and grew on a substrate of acetate but the reduction rate was less compared to that on other substrates.

Overall, the extent of Cr(VI) reduction correlated directly with bacterial growth. Furthermore, the rate of reduction of Cr(VI) generally decreased with time, regardless of the initial cell concentration. The decrease in the concentration of the utilizable organic substrate probably was an important reason for the decline in chromium reduction. In fact, respiking the same organic substrates (1% glucose, 1% lactate or 1% acetate) at 168 h during stationary phase increased the rate of growth in bacterial cultures containing 0.5 mM Cr(VI) (Figure 2).

As Figure 1 shows, we investigated the effect of initial Cr(VI) concentration on its rate of reduction over a Cr(VI) concentration range of 0.1 to 2.0 mM. Although the reduction of Cr(VI) by *Bacillus* sp. occurred even under the highest Cr(VI) concentration, it was not complete when the initial concentration was higher than 0.2 mM. The highest rate of Cr(VI) reduction was observed with initial concentrations between 0.1 to 0.2 mM with 1% glucose or 1% lactate as the sub strate; at these levels, Cr(VI) was completely reduced within 100 h (Figure 1).

Raising the concentration of Cr(VI) in the medium from 200 μM to 2.0 mM lowered the microbes' capacity for reduction and also lengthened the time taken for reduction to occur. Others also observed a similar trend with *Escherichia coli* (Shen & Wang 1994), *A. radiobacter* (Llovera et al. 1993), and *E. cloacae*

(Wang et al. 1989). The chromate ions must be transported by an active ion-pump mechanism inside the cell for the reduction to occur. This transport mechanism probably depends on some active membrane carriers which may become saturated or affected at higher concentrations of Cr(VI) affecting its transport. However, there may be other rate-limiting steps in the reductive process.

3.2 *Reduction of chromium (VI) by Bacillus strain in the presence of different concentrations of sulfate and thiosulfate*

We examined the growth and Cr(VI) reduction by *Bacillus* in the presence of two major sulfur species, sulfate and thiosulfate. The experiments were conducted at two different chromate values, 0.5 and 1.0 mM. Figure 3 compares bacterial growth and Cr(VI)

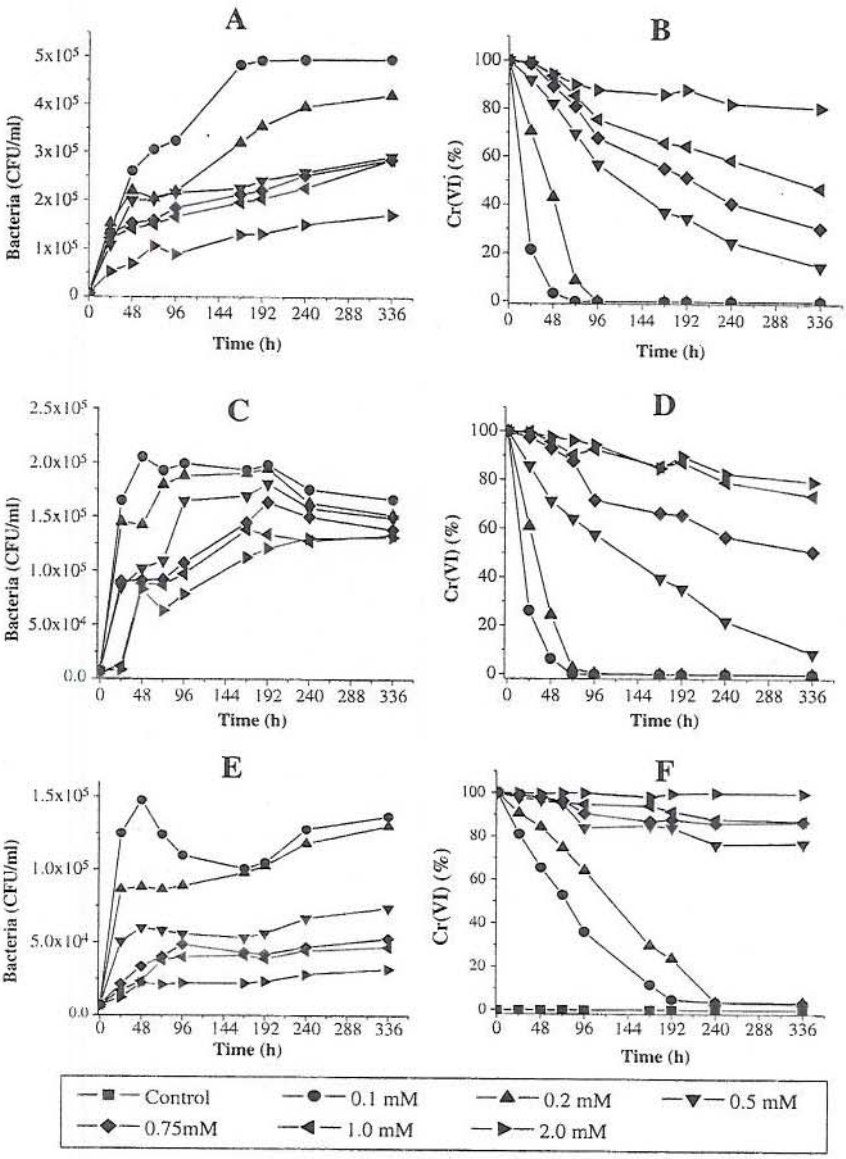

Figure 1. Growth of *Bacillus* sp. with different organic substrates in the presence of different concentrations of Cr(VI). A – Growth with glucose, B – Cr(VI) reduction with glucose, C – Growth with lactate, D – Cr(VI) reduction with lactate, E – Growth with acetate, F – Cr(VI) reduction with acetate.

reduction at two different sulfate concentrations of 0.05 mM and 1.0 mM in chromate concentration of 1.0 mM. Cr(VI) in the medium. As Figure 3 shows, varying the sulfate concentration in the media did not significantly affect the rate of reduction of Cr(VI). Similar results were obtained for 0.5 mM Cr(VI) in the medium. In contrast to sulfate, raising the thiosulfate concentration in the medium from 0.05 to 1.0 mM doubled the reduction rate under similar conditions (Figure 3). The bacterium reduced Cr(VI) more with thiosulfate present in the medium than with sulfate.

Table 1. Relative rates for chromium (VI) reduction by *Bacillus* sp. on different substrates.

Concentration of Cr(VI) (mM)	Relative rate of reduction ± standard error (μM/h)		
	Glucose	Lactate	Acetate
0.1	0.85 ± 0.14	0.83 ± 0.20	0.75 ± 0.27
0.2	1.48 ± 0.46	1.38 ± 0.54	1.14 ± 0.13
0.5	2.50 ± 0.13	2.15 ± 0.18	0.70 ± 0.16
0.75	2.70 ± 0.24	2.02 ± 0.31	0.82 ± 0.12
1.0	2.60 ± 0.31	0.90 ± 0.23	0.50 ± 0.12
2.0	2.20 ± 0.48	1.60 ± 0.46	0.20 ± 0.04

Overall, adding 1.0 mM thiosulfate greatly enhanced Cr(VI) reduction. QuiIntana et al. (2001) reported that adding elemental sulfur enhanced Cr(VI) reduction by *Thiobacillus ferroxidans*. They also demonstrated that the reducing compounds associated with colloidal sulfur reduce Cr(VI), even though their concentration in solution is low.

The reason for enhanced reduction of Cr(VI) in the presence of thiosulfate is not clear. In general, sulfur nucleophiles (e.g. thiols) are known to reduce Cr(VI) to Cr(III). Thus, the reduction of Cr(VI) can occur through a chemical reaction with thiosulfate.

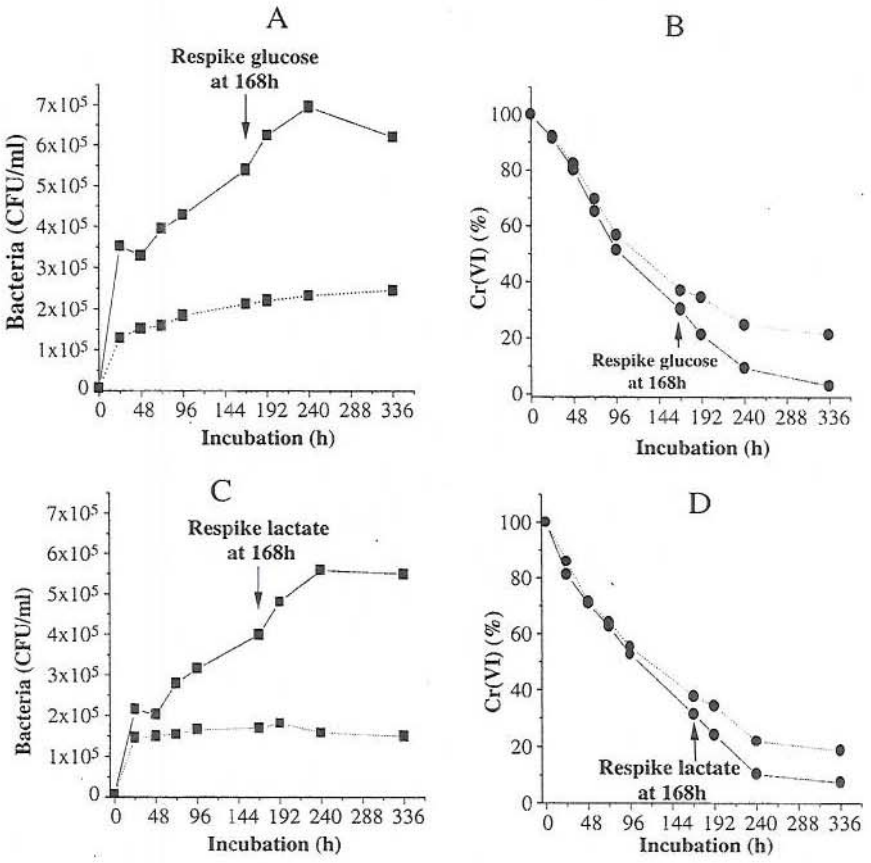

Figure 2. Effect of respiking with organic substrates during the stationary phase (~168 h) of growth and Cr(VI) reduction in comparison with a control that was not respiked. A – Growth curves with glucose; B – Cr(VI) reduction with glucose; C – Growth curves with lactate; D – Cr(VI) reduction with lactate.

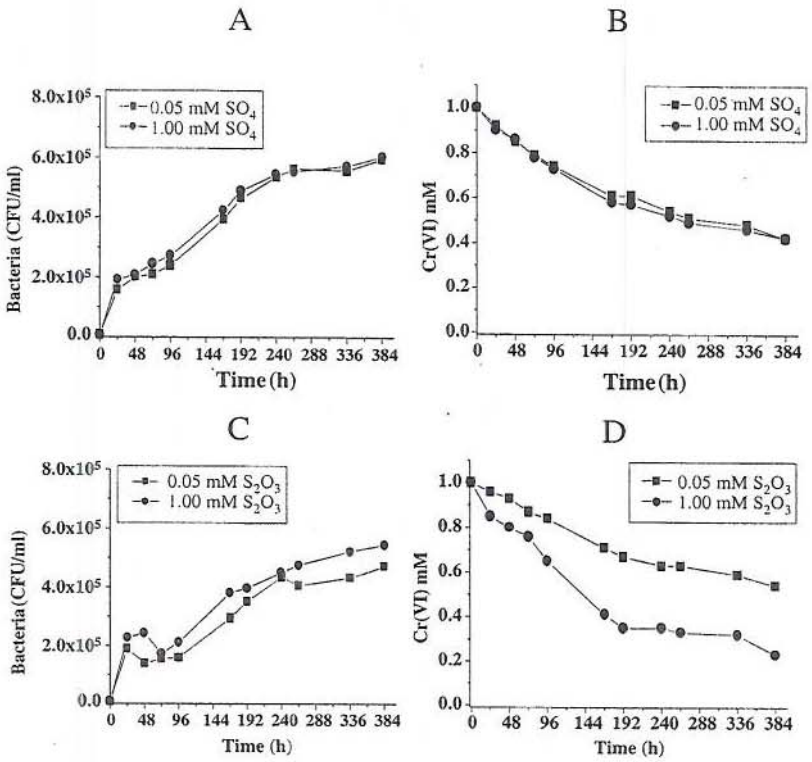

Figure 3. Effect of different concentrations of sulfate and thiosulfate on bacterial growth and reduction of 1.0 mM Cr(VI). A – Bacterial growth with sulfate, B -- Cr(VI) reduction with sulfate, C – Bacterial growth with thiosulfate, D – Cr(VI) reduction with thiosulfate.

However, as Figure 4 shows, the reduction of Cr(VI) was relatively insignificant in abiotic controls when compared to bacterial cultures (Figure 3). This result strongly suggests that biochemical reduction was the primary mechanism for the reduction of Cr(VI) in the presence of thiosulfate.

A possible mechanism for enhanced Cr(VI) reduction in the presence of thiosulfate may involve the uptake of thiosulfate and Cr(VI) by the bacteria, followed by a biochemically mediated reduction of the Cr(VI) by thiosulfate or any other reduced sulfur species generated from it. However, in general, biochemical reductions occur through mediation with reductase enzymes, which are mainly distributed in the cytoplasm. Because sulfhydryl sites are known to be active sites of several enzymes that mediate redox reactions (Miller et al. 1989, Rabuck et al. 1990, Ellis & Poole 1997), we suggest that these groups may also play a role in chromate reductases which convert Cr(VI) to Cr(III).

If this is the primary mechanism for Cr(VI) reduction in *Bacillus*, then our results may imply that when the bacteria grow in the presence of thiosulfate the biosynthesis of chromate reductase enzymes is enhanced.

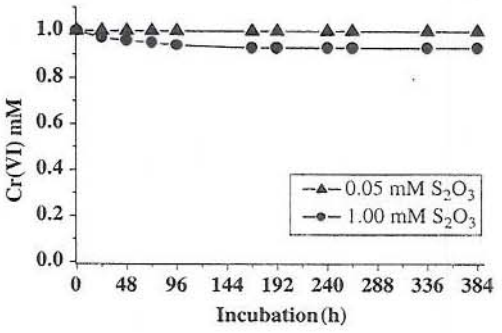

Figure 4. Cr(VI) reduction in abiotic controls for thiosulfate.

3.3 *Site of biochemical reduction of Cr(VI)*

We examined the cellular distribution of the reductases in the *Bacillus* sp. by monitoring the Cr(VI)-reducing

Table 2. Chromium (VI) reduction by cytoplasmic and cell membrane fractions.

	0.1 mM Cr(VI)		0.2 mM Cr(VI)	
Fraction	Reduction at 0.5 h	Reduction after 12 h	Reduction at 0.5 h	Reduction after 12 h
Membrane	6.4 ± 0.4[A]	10.3 ± 0.9	5.4 ± 0.2	10.6 ± 0.6
Cytoplasm	8.8 ± 0.9	16.9 ± 1.7	12.4 ± 0.8	20.0 ± 1.6

[A] Standard error

activity of isolated cell membranes and the cytoplasm fractions. Table 2 gives the relative efficiencies for Cr(VI) reduction by these different fractions at two different concentrations of Cr(VI). These results show that the enzymes mediating the reduction were mostly in the cytoplasm, although the cell membrane fractions show some activity.

Possibly, the membrane fraction was not completely separated from the soluble enzymes, rendering this effect. Enzymatic reduction of Cr(VI) has already been reported by many authors with different strains of microbes, such as *Bacillus* strain (Campos et al. 1995), *E. coli* (Shen & Wang 1994), *Pseudomonas maltophilia* (Blake et al. 1993), *Pseudomonas ambigua* (Suzuki et al. 1992), and in a species of *Pseudomonad* (McLean & Beveridge 2001). Our results obtained with a cytoplasmic and cell membrane fractions demonstrate that Cr(VI) reduction in *Bacillus* sp. reside mostly in soluble species localized in the cytoplasm, which probably are soluble reductases.

3.4 *Complex mechanism for Cr(VI) reduction*

The reduction of Cr(VI) mediated by reductase enzymes in aerobic bacteria such as *Bacillus* sp. seems to be a complex multistep process. Figure 5 summarizes a scheme of potential pathways for this reduction in the presence of thiosulfate.

It probably proceeds through a series of steps, of which the first should be an active uptake of the chromate ion by the bacteria. Uptake probably is concentration dependent and might be an important factor limiting the rate of reduction. Once Cr(VI) enters the cytoplasm, it may go through a chain of biochemical carriers, including the chromate reductase, to effect the reduction. Thiosulfate may enhance the reduction of Cr(VI) by accelerating the biosynthesis of chromate reductase enzymes, although there may be other mechanisms. The reduced species generated in the cytoplasm must be a soluble organic complex because the common Cr(III) species, Cr(III)-hydroxide, is insoluble and cannot be transported across the membrane. The Cr(III)-complex expelled into the extracellular medium then transforms to the insoluble hydroxide form.

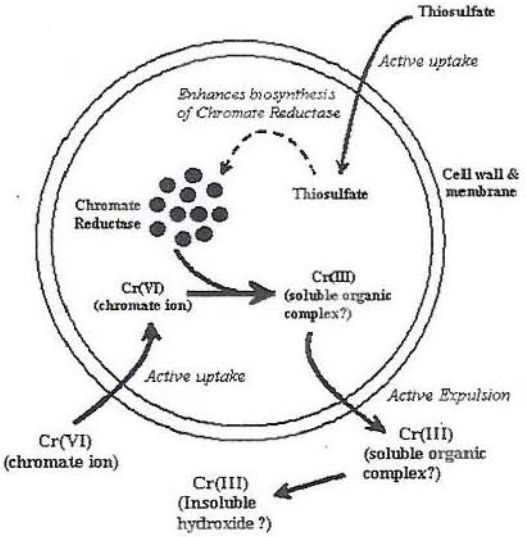

Figure 5. Scheme of possible pathways for Cr(VI) reduction in the presence of thiosulfate.

4 CONCLUSIONS

This work has shown that reductase enzyme extracted from the cell free broth can potentially be used as a stabilisation agent. Reductase enzyme reduces the toxic, soluble Cr(VI) to non-toxic insoluble Cr(III). The application of reductase enzyme in chromium contaminated sites for solidification would be a sustainable solution for remediation. Further, reductase enzyme production by bacteria could be enhanced using genetic manipulation techniques and research is needed to investigate its compatibility with conventional S/S using cement.

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REFERENCES

Allan, M.L. & KuKacka 1995. Blast furnace slag modified grouts for insitu stabilization of chromium contaminated soil. *Waste management* 15: 193–202.

APHA, 1989. Standard methods for the examination of water and wastewater. 17th Edition. American Public Health Association, Washington, D.C.

Beveridge, T.J. & Murray R.G.E. 1976. Uptake and retention of metals by cell walls of *Bacillus subtilis*. *Journal of Bacteriology* 127: 1502–1518.

Blake, R.C., Choate, D.M., Bardhan, S., Revis, N., Barton, L.L. & Zocco, T.G. 1993. Chemical transformation of metals by a *Pseudomonas* strain from a toxic waste site. *Environmental Toxicology & Chemistry* 12: 1365–1376.

Bopp, L.H. & Ehrlich, H.L. 1988. Chromate resistance and reduction in *Pseudomonas fluorescence* strain LB300. *Archives in Microbiology* 150: 426–431.

Campos, J., Martinezpacheco, M. & Cervantes, C. 1995. Hexavalent chromium reduction by a chromate-resistant *Bacillus* sp. strain. *Antonie Leeuwenhoek* 68: 203–208.

Dermatas, D. & Meng, X. 2003. Utilization of fly ash for stabilization/solidification of heavy metal contaminated soils. *Engineering Geology* 70: 377–394.

Ellis, H.R. & Poole, L.B. 1997. Roles for the two cystene residues of AhpC in catalysis of peroxide reduction by alkyl hydroperoxide reductase from *Salmonella typhimuriem*. *Biochemistry* 36: 13349–13356.

Fude, L., Harris, B., Urrutia, M.M. & Beveridge, T.J. 1994. Reduction of Cr(VI) by a consortium of sulfate reducing bacteria (SRBIII). *Applied & Environmental Microbiology* 60: 1521–1531.

Guha, H., Jayachandran, K. & Maurasse, F. 2001. Kinetics of chromium (VI) reduction by a type strain *Shewanella alga* under different growth conditions. *Environmental Pollution* 115: 209–218.

Gvozdyak, P.I., Mogilevich, N.F., Ryl'skii, A.F. & Grishchenko, N.I. 1986. Reduction of hexavalent chromium by collection strains of bacteria. *Microbiologiya* 55: 962–965.

Horitsu, H., Futo, S., Miyazawa, Y., Ogai, S., & Kawai, K. 1987. Enzymatic reduction of hexavalent chromium by hexavalent chromium tolerant *Pseudomonas ambigua* G-1. *Agricultural Biological Chemistry* 51: 2417–2420.

Idabacha, M.A., Nyavor, K. & Egiebor, N.O. 2003. Microbial stability evaluation of cement based waste forms at different waste to cement ratio. *Journal of Hazardous Materials* B96: 331–340.

Ishibashi, Y., Cervantes, C. & Silver, S. 1990. Chromium reduction in *Pseudomonas putida*. *Applied & Environmental Microbiology* 56: 2268–2270.

Kavasnikov, E.I., Stepanyuk, V.V., Klyushnikova, T.M., Serpokrylov, N.S., Simonova, G.A., Kasatkina, T.P. & Panchenko, L.P. 1985. A new chromium reducing gram variable bacterium with mixed type of flagellation. *Microbiologiya* 54: 69–75.

Kindness, A., Macias, A. & Glasser, F.P. 1994. immobilization of chromium in matrices. *Waste management* 14: 3–11.

Llovera, S., Bonet, R., Simon-Pujol, M.D. & Congregado, F. 1993. Chromate reduction by resting cells of *Agrobacterium radiobacter* EPS-916. *Applied & Environmental Microbiology* 59: 3516–3522.

Lovley, D.R. & Phillips, E.J.P. 1994. Reduction of chromate by *Desulfovibrio vulgaris* and its C_3 cytochrome. *Applied & Environmental Microbiology* 60: 726–728.

Lupton, F.S., DeFillippi, L.J. & Goodman, J.R. 1991. Bioremediation of chromium Cr(VI) contaminated aqueous systems by sulfate reducing bacteria. *U.S. Patent* 5,062956.

Mclean, J. & Beveridge, T.J. 2001. Chromate reduction by a *Pseudomonad* isolated from a site contaminated with chromated copper arsenate. *Applied & Environmental Microbiology* 67: 1076–1084.

McLean, J.S., Beveridge, T.J. & Philipps, D. 2000. Isolation and characterization of a chromium reducing bacterium from a chromated copper arsenate contaminated site. *Environmental Microbiology* 2: 611–619.

Mclean, J.S., Beveridge, T.J. & Philipps, D. 1999. Chromate removal from contaminated ground water using indigenous bacteria. In Alleman, B.C. & Leeson, A. (eds) *Proceedings of the In-situ and onsite bioremediation Fifth International symposium*. Batelle Press. 5: 121–126.

Miller, S.M., Moore, M.J., Massey, V., Williams, C.H., Distefano, M.D., Ballou, D.P. & Walsh, C.T. 1989. Evidence for the participation of Cys558 and Cys559 at the active site of mercuric reductase. *Biochemistry* 28: 1194–1205.

Otomosso, O.E., Ivey, D.G & Mikula, R. (1995). Electron microscopic studies of chromium doped tricalcium silicate. In Hager J.P., Misra, B., Davidson, C.F. & Litz, J.L.(eds.). *Treatment and minimisation of heavy metal containing wastes*, 129–141.

QuiIntana, M., Curutchet, G. & Donati, E. 2001. Factors affecting chromium (VI) reduction by *Thiobacillus ferroxidans*. *Biochemical Engineering Journal* 9: 11–15.

Rabuck, S.A., Distefano, M.D., Teo, B.K., Orme-Johnson, W. & Walsh, C.T. 1990. An EXAFS investigation of Hg(II) binding to mercuric reductase: comparative analysis of the wild-type enzyme and a mutant enzyme generated by site-directed mutagenesis. *Journal of American Chemical Society* 112: 1983–1989.

Rai, D., Sass, B.M. & Moore, D.A. 1987. Chromium (III) hydrolysis constants and solubility of chromium (III) hydroxide. *Inorganic Chemistry* 26: 345–349.

Romanenko, V.I. & Koren'kov, V.N. 1977. A pure culture of bacteria utilizing chromates and bichromates as hydrogen acceptors in growth under anaerobic conditions. *Microbiologiya* 46: 414–417.

Schmieman, E.A., Petersen, J.N., Yonge, D.R., Johnstone, D.L., Bereded S.Y., Apel, W.A. & Turick, C.E. 1997. Bacterial reduction of chromium. *Applied Biochemistry & Biotechnology* 63: 855–864.

Shen, H. & Wang, Y.T., 1993. Characterization of enzymatic reduction of hexavalent chromium by *Escherichia coli* ATCC 33456. *Applied & Environmental Microbiology* 59: 3771–3777.

Shen, H. & Wang, Y.T., 1994. Modeling hexavalent chromium reduction in *E.coli* 33456. *Biotechnology & Bioengineering* 43: 293–300.

Smillie, R.H., Hunter, K. & Loutit, M. 1981. Reduction of chromium(VI) by bacterially produced hydrogen sulfide in a marine environment. *Water Research* 15: 1351–1354.

Suzuki, T., Miyata, N., Horitsu, H., Kawai, K., Takamizawa, K., Tai, Y. & Okazaki, M. 1992. NAD(P)H-dependent chromium (VI) reductase of *Pseudomonas ambigua* G-1-A Cr(V) intermediate is formed during the reduction of Cr(VI) to Cr(III). *Journal of Bacteriology* 174: 5340–5345.

Tandon, S.K., Saxena, D.K., Gaur, J.S. & Chandra, S.V. 1978. Comparative toxicity of trivalent and hexavalent chromium. *Environmental Research* 15: 90–99.

Tebo, B.M. & Obraztsova, A.Y. 1998. Sulfate- reducing bacterium grows with Cr(VI), U(VI), Mn(IV) and Fe(III) as electron acceptors. *FEMS Microbiology Letters* 162: 193–198.

Wang, P.C., Mori, T., Komori, K., Sasatsu, M., Toda, K. & Ohtake, H. 1989. Isolation and characterization of *Enterobacter cloacae* strain that reduces hexavalent chromium under anaerobic conditions. *Applied & Environmental Microbiology* 55: 1665–1669.

Wang, Y.T. & Xiao, C. 1995. Factors affecting hexavalent chromium reduction in pure cultures of bacteria. *Water Research* 29: 2467–2474.